Phylogenetic relationships of African green snakes (genera *Philothamnus* and *Hapsidophrys*) from São Tomé, Príncipe and Annobon islands based on mtDNA sequences, and comments on their colonization and taxonomy

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Mitochondrial sequences (16S rRNA and cytochrome b) of the colubrine snake genera *Philothamnus* and *Hapsidophrys* were analysed. Samples were obtained from three volcanic islands in the Gulf of Guinea. The main objective was to infer phylogenetic relationships between the taxa and to trace back the colonization patterns of the group. Both insular species, *Philothamnus girardi* and *Philothamnus thomensis*, form a monophyletic unit indicating a single colonization event of one island (probably São Tomé) followed by dispersal to Annobon. Genetic divergence was found to be relatively low when compared with other *Philothamnus* species from the African mainland, but sufficient to consider the two taxa as distinct sister species. Here we also present evidence on the distinct phylogenetic position of *Hapsidophrys* sp. from the island of Príncipe, which should be considered as a distinct species, *Hapsidophrys principis*, a sister taxon of *H. smaragdina*.

Key words: 16S rRNA, cytochrome b, Gulf of Guinea islands, *Hapsidophrys principis*, *Philothamnus girardi*, *Philothamnus thomensis*

INTRODUCTION

The islands of the Gulf of Guinea form part of a volcanic chain that originated from the middle to late Tertiary. The islands São Tomé, Príncipe and Annobon are situated on the oceanic sector of a straight axis, the Cameroon volcanic line, which is a flaw or hotline (Meyers et al., 1998) in the African tectonic plate, about 1500 to 1600 km long (Déruelle et al., 1991; Burke, 2001; Caldeira & Munhá, 2002). In this volcanic line, the continental-shelf island Bioko is the largest and closest one to the African mainland; it is situated approximately 32 km from Cameroon and was formerly connected with the African mainland. The other three islands are truly oceanic ones; they are smaller than Bioko and were never connected with the mainland or with each other. Príncipe is situated about 220 km southwest from Bioko and 146 km northeast from São Tomé. The island of São Tomé is located about 275 km westwards from Gabon, while Annobon is about 180 km southwest of São Tomé. Príncipe (with an area of approximately 128 km²) has an estimated age of 31 million years, while the other islands are significantly younger: São Tomé (836 km²) is about 13 millions years old, and Annobon (17 km²) is apparently the youngest island with an age of about 4.9 million years (Lee et al., 1994).

The islands of the Gulf of Guinea belong biogeographically to the West African rainforest zone. They are situated between two large regions (the Guinea forests and the Congo basin) that have recently received increased attention due to their exceptional biodiversity. Based on their isolation, the islands presently harbour several endemic species. Moreover, the region could be considered one of the world’s hotspot for primates (Oates et al., 2004). As far as the herpetofauna are concerned, endemics on the islands are known among amphibians (Measey et al., 2007) and some reptiles: *Hemidactylus* geckos (Jesus et al., 2003, 2005a), *Lygodactylus* geckos (Jesus et al., 2006) *Mabuya* skinks (Jesus et al., 2003, 2005b,c), *Afroablepharus* skinks (Jesus et al., 2007), and various snakes (*Philothamnus thomensis*, *Philothamnus girardi* and *Hapsidophrys* spp.).

According to Chippaux (2001), about 19 snake species belong to the genus *Philothamnus*, distributed in Africa and mainly living in forests and riparian vegetation in sub-Saharan Africa. Despite comments made on a proposed revision (Hughes, 1985), the taxonomy of *Philothamnus* remains problematic (Trape & Roux-Estève, 1990).

*Philothamnus* and *Hapsidophrys* seem to constitute two closely related genera according to DNA sequence
data (Lawson et al., 2005). They are similar in appearance ("green snakes"), but differ in Hapsidophrys having strongly keeled dorsal scales. Despite the revision made by Hughes (1985), the taxonomy of the genus Philothamnus is still controversial (Trape & Roux-Estève, 1990).

Philothamnus thomensis Bocage, 1882 is endemic to São Tomé. It was treated by some authors (e.g. Mertens, 1934; Bogert, 1940; Loveridge, 1958) as a subspecies of P. semivariegatus (Smith, 1840), and has been confused with P. nitisus ( Günther, 1863). However, Hughes (1985) regarded it as a distinct species in his revision of the genus Philothamnus. Similarly, Philothamnus girardi Bocage, 1893 is endemic to Annobon and was also treated as a subspecies of P. semivariegatus by some authors, such as Mertens (1934) and Loveridge (1958). Furthermore, according to Hughes (1985) it shows some morphological resemblance to P. nitisus.

Hapsidophrys Fischer, 1856 is a small genus, and with the transfer of smaragdina from Gastropyxis it contains only two African species: H. lineatus Fischer, 1856 and H. smaragdina (Schlegel, 1837) (Broadley, 1966; Williams & Wallach, 1989; Chippaux, 2001). Gastropyxis principis was described by Boulenger (1906), and although sometimes still accepted as a valid species (e.g. Manaças, 1956), it has also been treated as a synonym of H. smaragdina (see, for example, Lawson et al., 2005). According to Meirte (1992), another taxon, Hapsidophrys coerules described by Fischer, 1856, from Ghana, has also been treated as a synonym of H. smaragdina.

### Table 1. Samples used in the present study. Abbreviations: PEM = Port Elizabeth Museum, Republic of South Africa; HLMD = Hessisches Landesmuseum Darmstadt, Germany.

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Despite recently published phylogenetic studies on the herpetofauna of the islands of the Gulf of Guinea (e.g. endemic amphibians: Drewes & Stoelting, 2004; Nesionixalus treefrogs: Drewes & Wilkinson, 2004; Ptychadena newtoni frog: Measey et al., 2007; Hemidactylus geckos: Jesus et al., 2005a; Lygodactylus geckos: Jesus et al., 2006; Mabuya skinks: Jesus et al., 2005b,c; and Afroablepharus skinks: Jesus et al., 2007), little is known about the snake fauna of the region. Whilst some studies indicate the monophyly of some reptile genera and probably a single colonization event – for example Lygodactylus (Jesus et al., 2006) and probably Afroablepharus (Jesus et al., 2007) – other studies indicate multiple colonization events, e.g. Mabuya (Jesus et al., 2005a,b) and Hemidactylus (Jesus et al., 2005). These results show remarkable variation between the relationships of taxa from different islands, indicating a complex pattern of colonization and dispersal.

In the present study, partial sequences from two mitochondrial genes, 16S rRNA and cytochrome b, were used to 1) study the phylogenetic relationships of green snakes from islands in the Gulf of Guinea; 2) examine the level of variation between populations and taxa on the islands; 3) infer the possible colonization events and patterns; and 4) infer the relationship of the insular species to other mainland species.

**MATERIALS AND METHODS**

**Sampling and molecular methods**

Locality and collection data on the specimens used in this study are given in Table 1 and Figure 1. Voucher specimens from São Tomé, Príncipe and Annobon are deposited in the herpetological/zoological collections of the University of Madeira. Further samples of the genera Philothamnus and Hapsidophrys are deposited in the Port Elizabeth Museum, South Africa.

Total genomic DNA was extracted using standard protocols (Sambrook et al., 1989). We used the following primers: 16SL and 16SH (Simon et al., 1990), and 16SA and 16SB (Palumbi et al., 1991) for amplification of 16S RNA, and cytB1 and CB3H (Palumbi et al., 1991), L14910, L14919 and H16064 (Burbrink et al., 2000; modified by de Queiroz et al., 2002) for amplification of cytochrome b. The primers cytochrome b2 from Kocher et al. (1989), P1 (see Jesus et al., 2007), L14903 (a 5’ end of L14910), L-410 and H-391 (Nagy et al., 2003) were used for sequencing of cytochrome b only. PCR protocols have been described elsewhere (Nagy et al., 2003; Jesus et al., 2007). PCR products were sequenced on ABI Prism 310 (Applied Biosystem) and MegaBACE 1000 (Amersham) capillary DNA sequencers.

DNA sequences were aligned using Clustal W (Thompson et al., 1994) followed by visual corrections.

The combined dataset used for phylogenetic analyses consisted of 1084 bp (447 bp for 16S RNA and 637 bp for cytochrome b). However, only cytochrome b sequences were used to estimate genetic divergence since this is comparable to many other studies.

Because phylogenetic reconstruction is based on positional homologies, the regions that could not be unambiguously aligned were excluded from further analysis (about 48 bp of 16S rRNA). The alignment is available on request from the corresponding author.

Six *P. thomensis* from São Tomé, one *P. girardi* from Annobon and three *Hapsidophrys* from Príncipe were compared with representatives of various *Philothamnus* species and both *H. lineatus* and *H. smaragdina* from the mainland (Table 1).

The false smooth snake *Macroprotodon cucullatus* was used as outgroup taxon (AY188026 for cytochrome b, AY188065 for 16S rRNA; see Table 1).

**Data analysis**

True evolutionary relationships may be obscured in DNA sequence data sets if sites have become saturated by multiple substitutions (Swofford et al., 1996). To test for saturation, observed pairwise proportions of transitions and transversions in the separate 16S and cytochrome b were plotted against sequence divergence using DAMBE version 4.2.13 (Xia & Xie, 2001).

Furthermore, differences in substitution rates between gene regions can produce conflicting signals. Thus, before proceeding with the analysis, a partition-homogeneity test was applied to our data (Farris et al., 1994) using PAUP* 4.0b10 (Swofford, 2002) to evaluate whether the two gene regions show significantly different phylogenetic signals. This test indicated no significant incongruence between regions (*P* = 0.692), so they were combined in all subsequent phylogenetic analyses.

Phylogenetic analyses were carried out using PAUP* 4.0b10 (Swofford, 2002) and MEGA version 3.1 (Kumar et al., 2004). We used maximum likelihood (ML) analyses and Bayesian inference of phylogeny. The most appropriate model of nucleotide substitution was inferred by Modeltest 3.7 (Posada & Crandall, 1998) and selected according to the Akaike information criterion (see Posada & Buckley, 2004). Non-parametric bootstrap support for nodes was estimated using the “fast” option with 100 heuristic bootstrap replicates as implemented in PAUP*.
Table 2. Cytochrome b K2P pairwise divergences for the *Philothamnus* and *Hapsidophrys* samples used in this study. Coding/abbreviations: *Ptho* = *Philothamnus thomensis*, *Pgir* = *Philothamnus girardi*, *Pang* = *Philothamnus angolensis*, *Pnat* = *Philothamnus natalensis*, *Pcar* = *Philothamnus carinatus*, *Pnit* = *Philothamnus nitidus*, *Phop* = *Philothamnus hoplogaster*, *Psem* = *Philothamnus semivariegatus*, *Phet* = *Philothamnus heterodermus*, *Haps* = *Hapsidophrys principis*, *Hlin* = *Hapsidophrys lineatus*, *Hsma* = *Hapsidophrys smaragdina*.

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The Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001). Bayesian analysis was conducted with random starting trees, four MCMC chains (one cold, three heated), run for 5 × 10⁶ generations, and sampled every 100 generations using a GTR+I+G model of nucleotide substitution. One additional analysis with 7.5 × 10⁶ generations was performed, leading to the same results. Two independent replicates were conducted and inspected for consistency (see Huelsenbeck & Bollback, 2001). Convergence between runs and posterior probabilities of the estimates were determined using the software Tracer (Rambaut & Drummond, 2005).

RESULTS

The dataset of the combined and aligned mtDNA sequences is comprised of 1084 bp from 22 specimens. Plots of observed pairwise divergences of haplotypes for transitions and transversions in the separate 16S rRNA and cytochrome b against total sequence divergence revealed negligible saturation (not shown), so our analyses included all sites. ML and Bayesian analyses gave identical topologies (Fig. 2). For the ML analysis we concluded that the GTR model (Rodríguez et al., 1990) with an estimate of invariable sites (0.62) and a discrete approximation of the gamma distribution (0.7497) was the most appropriate model. A heuristic search incorporating this model found one tree with a value of –lnL = 4658.

In all analyses, *Philothamnus thomensis* and *Philothamnus girardi* formed a monophyletic group supported by a posterior probability of 100% and a bootstrap value of 92%. This group is related to the group including *P. natalensis*, *P. carinatus*, *P. heterodermus* and *P. hoplogaster*. *Philothamnus* remains monophyletic with respect to *Hapsidophrys*, although only nine of the 19 recognized species of *Philothamnus* were used in our analysis.

All *Hapsidophrys*, including samples from Príncipe in the Gulf of Guinea and from the African mainland, form a monophyletic group supported by an average posterior probability of 100% and a bootstrap value of 86%.

Average levels of sequence divergence between congeneric reptile species are known to be approximately 12% for cytochrome b (Harris, 2002). We found a sequence divergence of 8.5% for cytochrome b between populations of *Philothamnus girardi* and *Philothamnus thomensis*. Sequence divergence between *Hapsidophrys* from Príncipe and *H. thomensis* and from the mainland was approximately 12%, and between *Hapsidophrys* from Príncipe and *H. lineatus* was approximately 12.5% (Table 2).

DISCUSSION

Colonization patterns

Our results clearly indicate that snakes belonging to the genus *Philothamnus* from Annobon and São Tomé are sister taxa. Despite the relatively low sequence divergence (8.5% for cytochrome b), they should be considered as distinct species, as proposed by Hughes (1985). Although monophyly of any group is dependent on outgroup sampling, the relatively low divergence between *P. thomensis* and *P. girardi* makes it less likely that any unsampled continental species will alter their status as sister species. Further, although sampling within islands is limited, genetic variation within both *P. thomensis* and *Hapsidophrys* from Príncipe is extremely low. Combined with the small size of the islands and the considerable dispersal ability of relatively large organisms such as these snakes, it seems unlikely that
additional genetically distinct units remain unsampled that might alter our colonization hypotheses. The ancestor of these species probably colonized one of these islands and then colonized the other. According to Emerson (2002), there are methods available to infer the colonization sequence. One uses tree topology and geography under the premise that an island is more likely to be colonized by a neighboring island than by a more distant one. The other method infers the direction of colonization using information from tree topology and branch length. This method is based on the assumption that there is a rapid molecular diversification caused by a founding event. So, taking into account the bigger branch length and longer distance of Annobon from the continent, the most parsimonious scenario seems to be that the first island that was colonized was São Tomé, followed by a radiation to Annobon. Measey et al. (2007) also suggest that São Tomé is a more probable target island to be first colonized, prior to Annobon, due to predicted rafting routes after river flooding on the continent.

This pattern seems to be similar to the case of Afroablepharus (Jesus et al., 2007), but differs from what was found in Mabuya skinks (Jesus et al., 2005c) and Hemidactylus geckos (Jesus et al., 2005a), which might have independently colonized each island.

When simply assuming a constant molecular clock for cytochrome b showing about 2% substitution rate per million years (see Carranza et al., 2000), the lineage inhabiting Annobon diverged from the lineage of São Tomé about 4.3 million years ago. This estimate is rather close to the genesis of Annobon. In fact, Annobon, the youngest and smallest of the Gulf of Guinea islands, has an estimated age of 4.9 million years (Lee et al., 1994).

Considering a similar molecular clock for Hapsidophrys, and assuming that the sister species of H. principis is H. smaragdina, the colonization might have been occurred about 6.1 million years ago from the mainland, or later from São Tomé from a lineage that has gone extinct meanwhile. However, the latter situation is less probable according to the hypothesis of Measey et al. (2007) about the direction of colonization on the Gulf of Guinea islands.

One additional aspect to be considered for the green snakes of these islands is that no island has multiple species. This is an observation that differs from the patterns found in Mabuya and Hemidactylus, where São Tomé harbors at least two species of each genus (Jesus et al., 2005a,c). It has been argued in other island systems that a filled ecological niche may reduce the success of further colonizations (Gillespie & Roderick, 2002), and this could

![Bayesian tree based on combined 16S rRNA and cytochrome b fragments. Posterior probabilities are shown above nodes. The tree was rooted using Macroprotodon cucullatus. The maximum likelihood tree, obtained by PAUP and using the GTR+I+G model of sequence evolution, shows identical topology. Bootstrap values (>50%) for ML are given below the nodes.](image-url)
explain the non-overlapping distribution of green snakes on these islands. As pointed out by Jesus et al. (2007), these differences in colonization patterns highlight the difficulties in drawing general conclusions regarding how islands are colonized by only a few species – stochastic processes obviously play an important role.

**Taxonomic comments**

Broadley’s (1966) action of synonymizing *Gastropyxix Cope, 1860* (type species: *Dendrophis smaragdina* Schlegel, 1837) with *Hapsidophrys* Fischer, 1856 (type species: *Hapsidophrys lineatus*) is supported.

The taxonomic status of *Hapsidophrys* snakes from Principe was controversially discussed in the past, including most recent references referring to them as *Hapsidophrys smaragdina* (e.g. Lawson et al., 2005). During most of the twentieth century, and even before 1906, these taxa were considered as synonyms. However, our results indicate high genetic divergences (based on partial cytochrome b sequences) between *H. smaragdina* and *Hapsidophrys* sp. from Principe, thus supporting the distinct status of the Principe population at species level. *Hapsidophrys principis* should therefore be considered as a valid species and not a synonym of *H. smaragdina*.

Described by Boulenger (1906) as *Gastropyxix principis*, it is represented by two syntypes stored in the Museo Civico di Storia Naturale, Genova, Italy (MSNG 28144a and MSNG 28144b) and one specimen in BMNH (according to the TIGR Reptile Database; Uetz et al., 2007).

Our molecular data also support specific status for *Philothamnus thomensis* and *P. girardi*, but do not indicate a close relationship with *P. semivariegatus* as proposed by Loveridge (1951, 1958), but questioned by Hughes (1985).

Our data indicate a close relationship between *P. semivariegatus* and *P. nitidus* (sequence divergence is approximately 11% – K2P distance), and of *P. carinatus* with *P. heterodermus* (sequence divergence is approximately 14%), as also found by Hughes (1985). Further studies, incorporating a wide range of continental samples of *Philothamnus* snakes, are needed to evaluate this topic.

**ACKNOWLEDGEMENTS**

This project was supported by grants from Fundação para a Ciência e Tecnologia (FCT) POCTI/41906/BSE/2001, POCTI/46647/BSE/2002. Fieldwork in the Gulf of Guinea was also supported by an award from the Gulbenkian society (to DJH). We would like to thank the Government of São Tomé and Principe and the Representative of Equatorial Guinea in São Tomé for permits to perform the field work. We acknowledge the practical support of the Joint Experimental Molecular Unit (JEMU). WRB thanks Conservation International (2001) and the Smithsonian Institution – Monitoring and Assessment of Biodiversity Program (2002) for the opportunity to work in Cote d’Ivoire and Gabon, respectively. Also, we would like to thank Dr Giuliano Doria, curator of the Museo Civico di Storia Naturale “Giacomo Doria” (Genova, Italy), for the precious information about the syntypes of *Hapsidophrys principis*.

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Systematic Biology 53, 793–808.


Accepted: 16 April 2009